REMARKS/ARGUMENTS

Claims 1-14 remain in this application. Claims 1 and 2 have been withdrawn from consideration. Claims 3-14 stand finally rejected under 35 U.S.C. § 103(a). Favorable reconsideration and examination of the subject application in light of the following remarks is respectfully requested.

Claim Rejections 35 USC § 103(a)

Claims 3-14 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Park, et al. (1999) in view of admitted prior art (Applicants' prior sale of cross-linked allophycocyanin which had not been exposed to strongly chaotropic agents). This rejection is respectfully traversed.

"Park, et al., disclose a method for quantitating an analyte by measuring time resolved fluorescence energy transfer to or from a label quantitatively associated with analyte." Page 3 of the Office Action. The Examiner acknowledges that Park fails to teach "cross-linked allophycocyanin [that] has not been exposed to strongly chaotropic agents after cross-linking.' Page 3 of the Office Action. However, Applicants placed cross-linked allophycocyanin which had not been exposed to strongly chaotropic agents on sale more than one year prior to the priority date of the present application, and the Examiner asserts that it would have been obvious to use the Applicants' "cross-linking agent" as an alternative to the "cross-linking agent of Park" and that both "cross-linking agents would have performed equally well." Page 3 of the Office Action. (Referral to "cross-linked" allophycocyanin in the subject application means a composition prepared as a reagent prior to the sale and use in an assay, not a cross-linking process that occurs as a step of an assay. Applicants assume that reference by the Examiner in the Office Action to "cross-linking agents" is intended as a reference to the cross-linked allophycocyanin product.)

In establishing obviousness, the prior art must teach or suggest the claimed invention. That a certain thing may result from a given set of circumstances is not sufficient to establish obviousness. *In re Oleich*, 212 U.S.P.O. 325, 326 (CCPA 1981). Further, the MPEP Eighth Edition § 2143.03 states that to establish prima facie obviousness all claim limitations must be taught or suggested by the prior art. However, no information was provided with Applicants' sale, or was reasonably ascertainable from Applicants' product at the time of sale, that would suggest or teach the use, in time-resolved fluorescence assays, of a reagent cross-linked allophycocyanin prepared by avoidance of the use of strongly chaotropic agents, nor was there any disclosure in the prior art that substitution of such a reagent into the method of Park could yield an improved time-resolved fluorescence assay.

The claims of the present application are directed to an improved method of measuring time-resolved fluorescence, the improvement relating to use of a reagent whose method of preparation was not publicly disclosed prior to the filing of the subject application. Use of a strongly chaotropic agent in the preparation of the reagent cross-linked allophycocyanin was the publicly known and accepted method at the time of the present invention, and not the avoidance of such reagents as taught in the present application's method of preparation of the cross-linked allophycocyanin. Further, no information was provided with the sale of Applicants' product, or available from examination of Applicants' product, which would suggest or teach that it had been prepared by avoiding strong chaotropic agents.

The Examiner asserts that both "cross-linking agents" [i.e. Applicants' cross-linked allophycocyanin reagent and the reagent of Park] would have performed equally well in time-resolved fluorescence assays." However, this statement is not correct. Applicants are claiming an improved assay. As pointed out in the Amendment filed May 10, 2004, Applicants' cross-linked

allophycocyanin provides an improvement over conventional cross-linked allophycocyanin when used in time-resolved fluorescence assay. It is well settled that a valid patent may issue for a non-obvious improvement on a prior patented invention, even though the improvement falls within the claims of the prior patent. See *Corning Glass Works v. Sumitomo Electric U.S.A.*, 868 F.2d 1251.

The obviousness analysis begins with a key legal question - what is the invention claimed? *Panderit Corp. v. Dennison Mfg. Co.*, 810 F.2d 1561, 1567 (Fed. Cir. 1987). Applicants are not generically claiming time-resolved fluorescence assays, but rather an improved time-resolved fluorescence assay where the improvement is related to use in the assay of a reagent prepared by a particular process. That process was contrary to methods known in the art and not ascertainable from physical examination of Applicants' product or sales-related public materials. Under the doctrine of slight changes originating in *Topliff v. Topliff*, 145 U.S. 156 (1982), slight modifications may be patentable. "[E]ven small differences in the art may establish patentability where the difference is distinctive, has great utility, and is not obvious." *Caterpillar Tractor Co. v. Berco*, S.P.A. 215 USPQ 948, 959 (D. Wyo. 1982), *aff'd on other grounds* 714 F.2d 1110 (Fed. Cir. 1983). Further, replacement of one material by another which yields surprising results is non-obvious. *See United States v. Adams*, 330 F.2d 622, 141 USPQ 361 (Ct. Cl. 1964).

In response to Applicants' argument that the skilled person would not be motivated to substitute Applicants' product for prior art allophycocyanin, the Examiner argues that Applicants' sale of the product was motivation, because the only possible use for the product was in an assay in accordance with Park, et al. On page 5 of the Office Action, the Examiner asserts that "there was no known or accepted use for cross-linked allophycocyanin other than for accepted assays [so] it would have been obvious to select [Applicants' cross-linked allophycocyanin] as an alternative for the [allophycocyanin] of Parks et al. [*sic*] ... Since the only known or accepted use of cross-linked

allophycocyanin purchased from Applicants would have been use in assays known in the art and therefore the methods would obviously yield improved analytical results and improved sensitivity."

The Examiner's statement is not correct, because Applicants' cross-linked allophycocyanin product may be used for standard binding assays (which are not claimed in the present application) with no difference in these binding assays from the binding assay results known in the prior art. The Examiner cites three articles which disclose the use of allophycocyanin in time resolved fluorescence assays. However, enclosed with this submission are nine (9) abstracts of papers published from 1994-2000 and describing use of allophycocyanin in flow cytometric assays which do not involve time resolved fluorescence. Clearly, time-resolved fluorescence was not the only know use for allophycocyanin.

Applicants' product was sold for use in a variety of assays other than time-resolved fluorescence assays. For example, Applicant's product may be used in a standard enzyme binding assays in which fluorescence, but not time resolved fluorescence, is measured. In the standard or non-time resolved assays, use of Applicants' product is not distinguishable from a prior art fluorescent labels, such as the allophycocyanin reagent used by Park. The mere fact that the product was sold does not provide motivation to select it for use in an improved time-resolved fluorescence assay or to expect that its use would yield the claimed improved time-resolved fluorescence assay. Further, Applicants' product can be used as a fluorescent label in qualitative assays such as trace labels in hazardous products to trace potential chemical spills. Accordingly, it is not inevitable that Applicant's product would have been used in a time-resolved fluorescence assay. Further, as discussed above, uses in standard enzyme binding assays, flow cytometry or as a qualitative tracer would not show improved results over use of cross-linked allophycocyanin prepared in the

conventional manner. Failure to find unexpected benefit upon use of the product in standard fluorescent assays provides no motivation to use the product in time-resolved fluorescence assays.

In determining prima facie obviousness it is necessary for one of ordinary skill in the art having the reference before him to be motivated make the proposed substitution, combination or other modification. *In re Lintner*, 458 F.2d 1013, 173 U.S.P.Q. 560 (CCPA 1972). Applicants submit that one skilled in the art with access to the references cited by the Examiner would not have any reason make the modification of using a reagent in a time-resolved fluorescence assay that had not been exposed to strong chaotropic agents during the process of preparing the reagent. Therefore, the invention claimed in the present application would not be prima facie obvious, and Applicants respectfully request that the rejection of claims 3-14 under 35 U.S.C. § 103(a) be withdrawn.

From the foregoing, reasonable action in the form of a Notice of Allowance is respectfully requested and earnestly solicited.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact the undersigned at the telephone number listed below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

The Commissioner is hereby authorized to charge payment or credit any overpayment to Deposit Account No. 50-0206 for any additional fee under 37 C.F.R. § § 1.16 or 1.17 required for reconsideration of this application as requested; particularly extension of time fees.

Respectfully submitted,

Date: September 1, 2004

By: _____
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APPENDIX A

1. Beavis A.J, Pennline K.J., "Detection of cell-surface antigens using antibody-conjugated fluorospheres (ACF): application for six-color immunofluorescence", Biotechniques (Sep 1996), Vol. 21, No. 3, pages 498-503.

2. Bigos M., Baumgarth N., Jager G.C., Herman O.C., Nozaki T., Stovel R.T., Parks D.R., Herzenberg L.A., "Nine color eleven parameter immunophenotyping using three laser flow cytomety", Cytometry (May 1, 1999), Vol. 36, No. 1, pages 36-45.

3. Corver W.E., Koopman L.A., van der Aa J., Regensburg M., Fleuren G.J., Cornelisse C.J., "Four-color multiparmeter DNA flow cytometric method to study phenotypic intratumor heterogeneity in cervical cancer", Cytometry (Feb 1, 2000), Vol. 39, No. 2, pages 96-107.

4. Donahue R.E., Kirby M.R., Metzger M.E., Agricola B.A., Sellers S.E., Cullis H.M., "Peripheral blood CD34+ cells differ from bone marrow CD34+ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor", Blood (Feb 15, 1996), Vol. 87, No. 4, pages 1644-1653.

5. Doornboos R.M., De Grooth B.G., Graan Y.M., Van Der Poel C.J., Greve J., "Visible diode lasers can be used for flow cytometric immunofluorescence and DNA analysis", Cytometry (Mar 1, 1994), Vol. 15, No. 3, pages 267-271

6. Gothot A., Grosdent J.C., Paulus J.M., "A strategy for multiple immunophentyping by image cytomety model studies using latex microbeads labeled with seven streptavidin-bound fluorichromes", Cytometry (Jul 1, 1996), Vol. 24, No. 3, pages 214-225.

7. Harding C.L., Lloyd D.R., McFarlane C.M., Al-Rubeai M., "Using the Microcycte flow cytometer to monitor cell number, viability, and apoptosis in mammalian cell culture", Biotechnol Prog. (Sep-Oct 2000), Vol. 16, No. 5, pages 800-802

8. Roederer M., De Rosa S., Gerstein R., Anderson M., Bigos M., Stovel R., Nozaki T., Parks D., Herzenberg L., Herzenberg L., "8 color, 10-parameter flow cytometry to elucidate complex leukocyte heterogeneity", Cytometry (Dec 1, 1997), Vol. 29, No. 4, pages 328-339.

9. Schmid I., Cole S.W., Zack J.A., Giorgi J.V., "Measurement of lymphocyte subset proliferation by three-color immunofluorescence and DNA flow cytometry", Immunol Methods (Feb 21, 2000), Vol. 235, No. 1-2, pages 121-131.

1: Biotechniques. 1996 Sep;21(3):498-503. Related Articles, Lin

Detection of cell-surface antigens using antibody-conjugated fluorospheres (ACF): application for six-color immunofluorescence.

Beavis AJ, Pennline KJ.

Hospital for Special Surgery, New York, NY, USA.

Antibody-conjugated fluorospheres (ACF) were used to phenotype murine leukocytes by flow cytometric analysis. Multicolor immunofluoresence (beyond simultaneous 4-color analysis) is limited by the availability of specific antibody-fluorochrome chrome conjugates and even further restricte by the spectral emission overlap of many of the fluorochromes when used in combination. Fluorospheres possessing unique excitation/emission spectra ca provide much needed versatility to existing protocols of multicolor fluorescence. SKY BLUE (647 nm excitation, 730 nm emission) fluorospher conjugated to CD11b monoclonal antibody were used in combination with th monoclonal antibodies IAd-FITC, L3T4 (CD4)-PE, LYT2 (CD8)-APC, THY1.2 (CD90)-biotin and B220 (CD45R)-RED613 for the simultaneous detection of six distinct cell-surface antigens in a mixed cell population. All fluorescence signals were resolved, and comparison of results from five-, six and single-color samples indicated that the percentages of cells positive for specific surface antigens were equivalent.

PMID: 8879591 [PubMed - indexed for MEDLINE]

1: Cytometry. 1999 May 1;36(1):36-45.

Nine color eleven parameter immunophenotyping using three laser flow cytometry.

Bigos M, Baumgarth N, Jager GC, Herman OC, Nozaki T, Stovel RT, Parks DR, Herzenberg LA.

Department of Genetics, Stanford University School of Medicine, California 94305-5318, USA. bigos@stanford.edu

BACKGROUND: This study describes a three laser flow cytometer, reagents and software used to simultaneously evaluate nine distinct fluorescent parameters on one cell sample. We compare the quality of data obtained with (1) full software compensation and (2) the use of partial spectral compensatic of selected pairs of parameters in analog hardware, in combination with final software compensation. An application characterizing low frequency murine cell subpopulations is given. METHODS: The fluorochromes used are: fluorescein (FITC), phycoerythrin (PE), Cy5PE and Cy7PE, excited at 488 n by an argon laser; Texas Red (TR), allophycocyanin (APC), and Cy7APC excited at 595 nm by a pumped dye laser; and cascade blue (CB) and cascade yellow (CY) excited at 407 nm by a violet-enhanced krypton laser. Custom additions to commercial electronics and an extended optical bench allow the measurement of these nine parameters plus forward and side scatter light signals. RESULTS: We find the use of partial analog compensation reduces the variation in the background staining levels introduced by the compensatic process. Novel B cell populations with frequencies below 1% are characterized. CONCLUSIONS: Nine color flow cytometry is capable of providing measurements with high information content. The choice of reager dye combinations and the ability to compensate in multi-parameter measurement space are crucial to obtaining satisfactory results.

PMID: 10331625 [PubMed - indexed for MEDLINE]

Jul 27 2004 06:47

1: Cytometry. 2000 Feb 1;39(2):96-107.

Four-color multiparameter DNA flow cytometric method to study phenotypic intratumor heterogeneity in cervical cancer.

Corver WE, Koopman LA, van der Aa J, Regensburg M, Fleuren GJ, Cornelisse CJ.

Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands. W.E.Corver@Pathology.MedFac.LeidentUniv.nl

BACKGROUND: Multiparameter DNA flow cytometry using a one-laser bench-top flow cytometer has been restricted to three different colors. The tw laser FACSCalibur has recently been introduced, allowing four-color analysi Therefore, we optimized and extended our three-color method (Corver et al., 1994, Corver et al. 1996) to a four-color analysis of phenotypic intra-tumor heterogeneity using a bench-top flow cytometer. METHODS: First, the effec of a range of different propidium iodide (PI) and TO-PRO-3 iodide (TP3) concentrations on the coefficient of variation (CV) of the DNA histograms was measured using paraformaldehyde-fixed lysolecithin-permeabilized peripheral blood lymphocytes (PBLs) and SiHa and HeLa cervical cancer cells. Second, labeling freshly isolated cervical cancers from solid tumors wa optimized with a mixture of anti-keratin antibodies. Third, the FACSCalibur hardware was modified, thereby allowing the simultaneous measurement of allophycocyanin (APC) fluorescence (FL4) in combination with FL3 pulse processing (FL3-W vs. FL3-A). The optimized procedure was then applied t cell suspensions from four different human cervical cancers to study phenotypic intratumor heterogeneity. Cell suspensions were simultaneously stained for DNA (PI, fluorescence) and three cellular antigens: (a) the epithelial cell-adhesion molecule (Ep-CAM; APC fluorescence), (b) keratin (R-phycoerythrin [RPE] fluorescence) to identify the epithelial fraction, and (c) vimentin (fluorescein-isothiocyanate [FITC] fluorescence) to label stroma cells. RESULTS: Overall, PI produced better CVs than did TP3. The optimal concentration of PI was 50-100 microM for all cells tested. Average CVs we 1.76% (PBL), 3.16% (HeLa), and 2.50% (SiHa). Optimal TP3 concentration were 0.25-2.0 microM. Average CVs were 2. 58% (PBL), 5.16% (HeLa), an 3.96% (SiHa). Inter- or intra-DNA stem line heterogeneity of Ep-CAM expression was observed in the keratin-positive fractions. Vimentin-positive, keratin-negative cells were restricted to the DNA diploid fraction.

CONCLUSIONS: PI is a superior DNA stain to TP3 when using intact norm PBL and human cancer cells. Four-color high-resolution multiparameter DN. flow cytometry allows the identification of intratumor subpopulations using I as DNA stain and FITC, RPE, and APC as reporter molecules. The FACSCalibur bench-top flow cytometer can be used for this purpose, allowir the application of this technique in clinical laboratories.

PMID: 10679727 [PubMed - indexed for MEDLINE]

1: Blood. 1996 Feb 15;87(4):1644-53. Related Articles, Lin

Peripheral blood CD34+ cells differ from bone marrow CD34+ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor.

Donahue RE, Kirby MR, Metzger ME, Agricola BA, Sellers SE, Cullis HM.

Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20850, USA.

Granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) have been shown to stimulate the circulation of hematopoietic progenitor cel in both mice and nonhuman primates. We evaluated the immunophenotype and cell cycle status of CD34+ cells isolated from the bone marrow (BM) an leukapheresis product of cytokine-mobilized nonhuman primates. CD34+ cel were isolated from rhesus macaques that had received no cytokine therapy, 100 micrograms/kg/d G-CSF, 200 micrograms/kg/d SCF, or a combination o both 100 micrograms/kg/d G-CSF and 200 micrograms/kg/d SCF as a subcutaneous injection for 5 days. BM was aspirated before (day 0) and on th last day (day 5) of cytokine administration. On days 4 and 5, peripheral bloo (PB) mononuclear cells were collected using a novel method of leukapheresi Threefold more PB mononuclear cells were collected from animals receiving G-CSF alone or G-CSF and SCF than from animals that had received either SCF alone or no cytokine therapy. CD34+ cells were positively selected usin an immunoadsorptive system from the BM, PB, and/or leukapheresis produc Threefold and 10-fold more CD34+ cells were isolated from the leukapheres product of animals receiving G-CSF or G-CSF and SCF, respectively, than from animals receiving no cytokine therapy or SCF alone. The isolated CD3 cells were immunophenotyped using CD34-allophycocyanin, CD38-fluorescein isothiocyanate, and Thy-1-phycoerythrin. These cells were later stained with 4', 6-diamidino-2-phenylindole for simultaneous DNA analysis and immunophenotyping. BM-derived CD34+ cells did not differ significant in cell cycle status and Thy-1 or CD38 phenotype before or after G-CSF and/or SCF administration. Similarly, CD34+ cells isolated from the leukapheresis product did not differ significantly in immunophenotype or cel cycle status before or after G-CSF and/or SCF administration. However, ther were consistent differences in both immunophenotype and cell cycle status

between BM- and PB-derived CD34+ cells. CD34+ cells isolated from the Pl consistently had a smaller percentage of cells in the S+G2/M phase of the cel cycle and had a higher percentage of cells expressing Thy-1 than did CD34+ cells isolated from the BM. A greater proportion of PB-derived CD34+ cells were in the S+G2/M phase of the cell cycle after culture in media supplemented with interleukin-6 and SCF, However, culturing decreased the proportion of CD34+ cells expressing Thy-1.

PMID: 8608259 [PubMed - indexed for MEDLINE]

1: Cytometry. 1994 Mar 1;15(3):267-71. Related Articles, Lin

Visible diode lasers can be used for flow cytometric immunofluorescence and DNA analysis.

Doornbos RM, De Grooth BG, Kraan YM, Van Der Poel CJ, Greve J.

Applied Optics Group, Faculty of Applied Physics, University of Twente, Enschede, The Netherlands.

This report describes a feasibility study concerning the use of a visible diode laser for two important fluorescence applications in a flow cytometer. With a mW 635 nm diode laser, we performed immunofluorescence measurements using the fluorophore allophycocyanin (APC). We have measured CD8 positive lymphocytes with a two-step labeling procedure and the resulting histograms showed good separation between the negative cells and the dim and the bright fluorescent subpopulations. As a second fluorescence application, we chose DNA analysis with the recently developed DNA/RNA stains TOTO-3 and TO-PRO-3. In our setup TO-PRO-3 yielded the best results with a CV of 3.4%. Our results indicate that a few milliwatts of 635 n light from a visible diode laser is sufficient to do single color immunofluorescence measurements with allophycocyanin and DNA analysis with TO-PRO-3. The major advantages of using a diode laser in a flow cytometer are the small size, the low price, the high efficiency, and the long lifetime.

PMID: 8187585 [PubMed - indexed for MEDLINE]

1: Cytometry. 1996 Jul 1;24(3):214-25. Related Articles, Lin

A strategy for multiple immunophenotyping by image cytometr model studies using latex microbeads labeled with seven streptavidin-bound fluorochromes.

Gothot A, Grosdent JC, Paulus JM.

Laboratory of Hematology, Hopital du Sart-Tilman and University of Liege, Liege, Belgium.

Multiple immunophenotyping is aimed at identifying several cell populations in a single labeling procedure by their ability to bind combinations of specifi labeled antibodies. The present work demonstrates the simultaneous discrimination by using image cytometry of aminomethylcoumarin acetate (AMCA), Lucifer yellow (LY), fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), PE-Texas red tandem (Red613), peridinin-chlorophyll protein (PerCP), and allophycocyanin (APC), which were all bound to latex beads as streptavidin-conjugated fluorochromes. This has been the result of a step-by-step optimization of the several factors affecting the sensitivity and specificity of multiple immunofluorescence analysis. First, 14 streptavidin-conjugated fluorochromes were evaluated by using spectrofluorometry. A primary selection was then made of ten spectrally separable dyes that could b evaluated by using image cytometry. These dyes were bound to latex particle and specific filter combinations were assembled to minimize crosstalk between fluorophores while preserving sufficient fluorescence intensity and counting statistics. Potential probe associations were then assessed by measuring the emissions of all fluorochromes that were detected by each filte combination. The resulting crosstalk matrix served as the basic tool both for final selection of the optimal filter combination and for dye set (composed, ir this case, of the seven fluorochromes described above) and for mathematical correction of residual spectral overlap. Next, an image cytometry system was adapted to collect seven images of matched brightness with the selected combination of excitation/emission filters and dichroic mirrors. Finally, seve parameter synthetic images were generated by digital image processing.

PMID: 8800554 [PubMed - indexed for MEDLINE]

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1: Biotechnol Prog. 2000 Sep-Oct;16(5):800-2.

Using the Microcyte flow cytometer to monitor cell number, viability, and apoptosis in mammalian cell culture.

Harding CL, Lloyd DR, McFarlane CM, Al-Rubeai M.

Aber Instruments Ltd., Science Park, Aberystwyth, SY23 3AH, U.K.

The Microcyte is a novel, portable flow cytometer based on diode laser technology whose use has been established for yeast and bacterial analysis. We present data that demonstrate its suitability for routine mammalian cell counting and viability determination. To extend its range of applications in tl field of animal cell culture biotechnology, a test to determine the number of apoptotic cells present has been developed for use with the instrument. Apoptosis was induced in hybridoma cell cultures by treatment with camptothecin. Apoptotic cells were labeled with biotinylated Annexin V and then visualized using a streptavidin-allophycocyanin conjugate. Their numbe were counted, and the cell size of the apoptotic cell population was determin using the Microcyte.

PMID: 11027173 [PubMed - indexed for MEDLINE]

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1: Cytometry. 1997 Dec 1;29(4):328-39. Related Articles, Lin

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8 color, 10-parameter flow cytometry to elucidate complex leukocyte heterogeneity.

Roederer M, De Rosa S, Gerstein R, Anderson M, Bigos M, Stovel R, Nozaki T, Parks D, Herzenberg L, Herzenberg L.

Department of Genetics, Stanford University, California, USA.
roederer@darwin.stanford.edu

We developed the chemistry, instrumentation, and software technologies needed to measure, simultaneously and independently, eight different fluorescent molecules on individual cells. Conjugation of these fluorochrome to monoclonal antibodies is straightforward; all immunofluorescence staining is accomplished with direct stains only. We built a hybrid flow cytometer wit eight fluorescence detectors and two light scatter channels, with excitation provided by three spatially separated laser beams emitting at 407 nm, 488 nn and 595 nm. The fluorescence compensation required to make the data orthogonal is of sufficient complexity that it cannot be performed manually; thus, we use software to compensate the data post hoc, based on data collecte from singly stained compensation control samples. In this report, we evaluate the 8 color staining technology. Of the seven fluorochromes other than fluorescein, six have a useful brightness at least as great as fluorescein. Three of the fluorochromes (phycoerythrin, allophycocyanin, and the Cy5 resonanc energy tandem of phycoerythrin) are considerably brighter than fluorescein and are useful for detecting antigens expressed at low levels. Finally, we sho the power and utility of the 8 color, 10-parameter technology using staining experiments on both human and murine immune systems.

PMID: 9415416 [PubMed - indexed for MEDLINE]

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1: J Immunol Methods. 2000 Feb 21;235(1-2):121-31. Related Articles, Lin

Measurement of lymphocyte subset proliferation by three-color immunofluorescence and DNA flow cytometry.

Schmid I, Cole SW, Zack JA, Giorgi JV.

UCLA School of Medicine, Department of Hematology/Oncology, 12-236 Factor Building, Los Angeles, CA 90095, USA. schmid@mednet.ucla.edu

We developed a method for simultaneous flow cytometric analysis of three-color immunofluorescence and DNA content. We show here that staining wit 7-amino-actinomycin D (7-AAD) at 10 microg/ml using a phosphate-citrate buffer at low pH containing saponin for cell membrane permeabilization yields good resolution DNA histograms with low coefficients of variation. Furthermore, light scatter properties of cells are preserved after permeabilization; this permits gating on cell populations that differ in scatter signals on the flow cytometer. Because of the low pH of the phosphate-citrat staining buffer, Alexa488, a pH-independent green-fluorescent fluorochrome is used instead of fluorescein-isothiocyanate (FITC) for cell surface staining combination with phycoerythrin (PE) and with allophycocyanin (APC) whicl are both pH insensitive. Removal of 7-AAD after staining and replacing it with non-fluorescent actinomycin D (AD) retains DNA staining and allows detection of Alexa488, PE and APC cell surface immunofluorescence withou interference from fluorescent 7-AAD in solution for clear identification of ce subpopulations even after prolonged stimulation in culture. Thus, using a fou color benchtop flow cytometer, measurement of Alexa488, PE and APC thre color immunofluorescence can be combined with 7-AAD DNA content analysis. Furthermore, we demonstrate that sample storage overnight without fixation for later analysis on the flow cytometer is possible without compromising results. Application of the method to the assessment of the differential proliferative responses of lymphocyte subsets of human peripher blood mononuclear cells (PBMC) that were costimulated with CD3 and with CD28.2 is presented.

PMID: 10675764 [PubMed - indexed for MEDLINE]

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